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δ-tocotrienol protects mouse and human hematopoietic progenitors from γ-irradiation through Erk/mTOR signaling

by Xiang Hong Li, Dadin Fu, Nabil H. Latif, Conor P. Mullaney, Patrick H. Ney, Steven R. Mog, Mark H. Whitnall, Venkataraman Srinivasan, and Mang Xiao

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14. ABSTRACT

Background and Objectives. Exposure to "-radiation causes rapid hematopoietic cell apoptosis and bone marrow suppression. However, there are no approved radiation countermeasures for the acute radiation syndrome. In this report, we demonstrate that natural delta-tocotrienol, one of the isomers of vitamin E, significantly enhanced survival in total body lethally irradiated mice. We explored the effects and mechanisms of delta-tocotrienol on hematopoietic progenitor cell survival after " -irradiation in both in vivo and in vitro experiments. Design and Methods. CD2F1 mice and human hematopoietic progenitor CD34+ cells were treated with delta-tocotrienol or vehicle control 24 h before or 6 h after " -irradiation. Effects of delta-tocotrienol on hematopoietic progenitor cell survival and regeneration were evaluated by clonogenicity, flow cytometry, and bone marrow histochemical staining. Delta-tocotrienol and "-irradiation-induced signal regulatory activities were assessed by immunofluorescence staining immunoblotting and siRNA assay. Results. Delta-tocotrienol displayed significant radioprotective effects. A single injection of delta-tocotrienol protected 100% of CD2F1 mice from total body irradiation-induced death as measured by 30-day post-irradiation survival. Delta-tocotrienol increased cell survival, and regeneration of hematopoietic microfoci and lineage-negative/Sca-1-positive/ckit-positive stem and progenitor cells in irradiated mouse bone marrow, and protected human CD34+ cells from radiation?induced damage. Delta-tocotrienol activated Erk1/2 phosphorylation and significantly inhibited formation of DNA-damage marker "-H2AX foci. In addition, delta-tocotrienol upregulated mTOR and phosphorylation of its downstream effector 4EBP-1. These alterations were associated with activation of mRNA translation regulator eIF4E and ribosomal protein S6 which is responsible for cell survival and growth. Inhibition of Erk1/2 expression by siRNA abrogated delta-tocotrienol-induced mTOR phosphorylation and clonogenicity, and increased "- H2AX foci formation in irradiated CD34+ cells. Interpretation and Conclusions. Our data indicate that delta-tocotrienol protects mouse bone marrow and human CD34+ cells from radiation-induced damage through Erk activationassociated mTOR survival pathways.

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Abstract

Background and Objectives. Exposure to γ-radiation causes rapid hematopoietic cell apoptosis and bone marrow suppression. However, there are no approved radiation countermeasures for the acute radiation syndrome. In this report, we demonstrate that natural delta-tocotrienol, one of the isomers of vitamin E, significantly enhanced survival in total body lethally irradiated mice. We explored the effects and mechanisms of delta-tocotrienol on hematopoietic progenitor cell survival after γ -irradiation in both *in vivo* and *in vitro* experiments.

Design and Methods. CD2F1 mice and human hematopoietic progenitor CD34+ cells were treated with delta-tocotrienol or vehicle control 24 h before or 6 h after γ -irradiation. Effects of delta-tocotrienol on hematopoietic progenitor cell survival and regeneration were evaluated by clonogenicity, flow cytometry, and bone marrow histochemical staining. Delta-tocotrienol and γ-irradiation-induced signal regulatory activities were assessed by immunofluorescence staining, immunoblotting and siRNA assay.

Results. Delta-tocotrienol displayed significant radioprotective effects. A single injection of delta-tocotrienol protected 100% of CD2F1 mice from total body irradiation-induced death as measured by 30-day post-irradiation survival. Delta-tocotrienol increased cell survival, and regeneration of hematopoietic microfoci and lineage-negative/Sca-1-positive/ckit-positive stem and progenitor cells in irradiated mouse bone marrow, and protected human CD34+ cells from radiation–induced damage. Delta-tocotrienol activated Erk1/2 phosphorylation and significantly inhibited formation of DNA-damage marker γ-H2AX foci. In addition, delta-tocotrienol

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upregulated mTOR and phosphorylation of its downstream effector 4EBP-1. These alterations were associated with activation of mRNA translation regulator eIF4E and ribosomal protein S6, which is responsible for cell survival and growth. Inhibition of Erk1/2 expression by siRNA abrogated delta-tocotrienol-induced mTOR phosphorylation and clonogenicity, and increased γ-H2AX foci formation in irradiated CD34+ cells.

Interpretation and Conclusions. Our data indicate that delta-tocotrienol protects mouse bone marrow and human CD34+ cells from radiation-induced damage through Erk activation-associated mTOR survival pathways.

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Introduction

More than 50% of cancer patients receive radiotherapy, which often results in side effects due to radiation damage on normal tissue,[1] such as bone marrow failure (BMF) syndrome.[2] The hematopoietic system is among the most radiation-sensitive organs.[3] A dose of ionizing radiation (IR) above 1 Gy in humans poses a risk of injury to the bone marrow and hematopoietic system, which leads to long-term compromised immune function and increased susceptibility to infection and internal and external hemorrhage.[4] The mechanism of IR-induced BMF is not well understood and there are no FDA-approved pharmaceuticals to counter acute radiation syndrome. In this study, we demonstrate that delta-tocotrienol (DT3), one of the isomers of vitamin E, has significant radioprotective effects on lethal total body _-irradiated (TBI) mice as measured by 30-day post-irradiation survival. DT3 rescued mouse and human hematopoietic stem and progenitor cells from radiation damage.

There are eight distinct analogs of vitamin E designated α-, β-, γ-, and δ-tocopherol; and α-, β-, γ-, and δ-tocotrienol.[5, 6] Previous studies have been conducted with tocopherols, the most commonly used vitamin E supplement and the most abundant vitamin E isoforms in human and animal tissue. During the last decade, tocotrienol research has gained substantial momentum. Tocotrienols have shown neuroprotective, anticancer, anti-oxidative, and cholesterol-decreasing effects that are often not exhibited by tocopherols.[5-7] The present study evaluated the mechanism of radioprotection by DT3. We found DT3-induced extracellular signal-regulated kinase 1/2 (Erk1/2) and mammalian target of rapamycin (mTOR) activation in human hematopoietic CD34+ cells (*in vitro*) and mouse bone marrow (*in vivo*) was associated with increased cell survival, and decreased formation of the radiation-induced DNA-damage marker γ-H2AX foci.

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Erk1/2 [8] activity is a component of the pro-survival mitogen-activated protein kinase (MAPK) pathway, which induces expression of DNA repair and cell growth factors.[9-11] mTOR is a member of the phosphatidylinositol 3-kinase (PI3K)-related kinase family and plays a central role in regulating protein synthesis, ribosomal protein translation, and cap-dependent translation,[12] which are involved in cell proliferation, cell cycle progression, DNA damage checkpoints, and cell survival and growth.[13] Major upstream signaling components that control mTOR activity are the PI3K/Akt and Erk/MAPK pathways,[14, 15] and the immediate mTOR downstream targets are eukaryotic initiation factor 4E (eIF4E)-binding protein-1 (4EBP-1) and the ribosomal protein S6 kinase (S6K).[16] 4EBP1 and S6K activity are controlled by mTOR, which phosphorylates and inactivates 4EBP1 (the inhibitor of eIF4E), and phosphorylates and activates S6K, resulting in stimulation of cap-dependent mRNA translation in eukaryotic cells. Most mRNAs are translated in mammalian cells through a cap-dependent mechanism,[17] and translational control allows for rapid protein regulation in response to positive or negative stimuli including cell growth, cell cycle progression, and apoptosis.[18] In the present study, we investigated the effects of DT3 as a radiation countermeasure and the mechanisms of DT3-induced Erk1/2 and mTOR signaling.

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Design and Methods

Mice

Twelve to 14 week old CD2F1 male mice (Harlan Laboratories, Indianapolis, IN) were used for survival studies according to methods described in previous reports.[19] Animals were housed in an AAALAC-approved facility at the Armed Forces Radiobiology Research Institute (Bethesda, MD). The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

Human CD34+ cells

Human hematopoietic CD34+ cells were provided by the National Hematopoietic Cell Processing Core, Fred Hutchinson Cancer Research Center (Seattle, WA). CD34+ cells were cultured in serum-free medium consisting of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with BIT 9500 (Stem Cell Technologies, Tukwila, WA) and penicillin/streptomycin. Recombinant human (rh) stem cell factor (SCF, 100 ng/ml), rh flt-3 ligand (FL, 100 ng/ml), and rh interleukin-3 (IL-3, 25 ng/ml) were added. All cytokines were purchased from PeproTech, Inc. (Rocky Hill, NJ). CD34+ cells were incubated at 37^{o}C with 5% CO_2.

Drug and irradiation

Delta-tocotrienol (DT3) was purchased from Yasoo Health Inc., Johnson City, TN. The drug was solubilized in PEG-400 and 5% Tween-80 for *in vivo* studies, while the drug was dissolved in ethanol for *in vitro* studies. A vehicle control consisting of PEG-400 and 5% Tween-80 was used for animal studies. Ethanol was used as a control in *in vitro* studies.

DT3 (400 mg/kg) or vehicle was administered as a single subcutaneous (sc) dose to mice 24 h before (-24 h) or 6 h after (+6 h) TBI at doses of 0 (sham-irradiation), 5.0, or 8.75 Gy, at a dose rate of 0.6 Gy/min in the Institute's cobalt facility. Sham-irradiated mice were treated exactly the same way as the γ-irradiated animals except the cobalt-60 source was not raised from the shielding water pool. After irradiation, mice were returned to their home cages. The day of irradiation was regarded as day 0. Survival was monitored for 30 days post-irradiation.

For the *in vitro* study, DT3 (2 μM) or vehicle control (alcohol) was added to the human CD34+ cell culture 24 h before exposure to γ-irradiation at doses of 0, 2, or 4 Gy (0.6 Gy/min).[20] After irradiation, cells were washed once and cultured in fresh culture medium without DT3. For the +6 h treatment groups, the same dose of DT3 or vehicle was added post-irradiation. Total survival cell number after irradiation was counted by Trypan blue staining.

Pathology of mouse bone marrow

Mouse sternums were fixed in Z-Fix (formaldehyde, methanol, ionized zinc buffer, Anatech Ltd, Battle Creek, MI, USA) for at least 24 h. Samples were decalcified (Cal-EX for 3 h) and sectioned longitudinally for hematoxylin-eosin (HE) staining. Slides were first examined at 20x. Bone marrow cellularity was measured by a subjective analysis of 8-14 adjacent low power

(200x) microscopic fields for each sectioned sternum (4-6 sternebrae). Megakaryocytes were evaluated by a subjective analysis of three adjacent high power (600x) microscopic fields for each sternebra.

Mouse bone marrow myeloid cell viability and cell phenotype analysis

Bone marrow cells were collected from mouse femurs and humeri 1, 8 and 13 days after TBI. After erythrocytes were lysed by erythrocyte lysis buffer (Qiagen GmbH, Hilden), total bone marrow myeloid cell viability for pooled samples from each mouse was measured by Trypan blue staining and by BD FACSCalibur flow cytometry analysis after labeling with Annexin-V (apoptotic cell marker) and 7-aminoactinomycin D (7AAD, a death marker). Total live myeloid cell numbers from individual mice were measured on days 1, 8 and 13 after TBI or sham-irradiation. Phenotypes of mouse bone marrow cells were quantified using the BD FACSCalibur. Cells were gated for 7AAD-positive dead cells and negative live cells. Mouse lineage, c-kit, and Sca-1 antibodies were used for phenotype determination in 7AAD-negative populations. All antibodies and dyes were purchased from BD Biosciences (San Jose, CA, USA).

Mouse BM cell and human CD34+ cell clonogenic assay

Clonogenicity of mouse bone marrow cells and human CD34+ cells was quantitated in standard semisolid cultures in triplicate using 1 ml of Methocult GF+ system for either mouse cells or human cells (Stem Cell Technologies) according to the manufacturer's instructions, as described previously.[20] Briefly, mouse bone marrow cells from pooled samples or CD34+ cells from liquid culture were washed twice with IMDM and seeded at 1-5 x 10^4 cells/dish (mouse cells) or 1-5 x 10^3 cells/dish (CD34+ cells) in 35-cm cell culture dishes (from BD Biosciences). Plates were

scored for erythroid, granulocyte-macrophage, and mixed-lineage colonies after culturing for 10 days (for mouse colonies) or 14 days (for human colonies) at 37°C, 5% CO_2.

Immunoblotting

1-5 x 10^6 cells from each sample were harvested, washed, and lysed with 1x Laemmli sample buffer. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Immunoblotting was performed following standard procedures with an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL) and Kodak X-ray film. Antibodies for Erk1/2, phosphorylated Erk1/2 (p-Erk1/2), Akt, p-Akt, mTOR, p-mTOR, 4EBP1, p-4EBP1, eIF4E, p-eIF4E, S6, and p-S6 were from Cell Signaling (Danvers, MA, USA).

Erk siRNA transfection

SignalSilence Erk1 and Erk2 siRNA from siGENOME SMARTpool (1.5 μg each, Dharmacon Inc., Lafayette, CO) or 1.5 μg of maxGFP siRNA (positive control provided in the siRNA Test Kit, amaxa, Inc.) were transfected into 3x10^6 human CD34+ cells using the Human CD34 Cell Nucleofector Kit and Nucleofector II (amaxa Inc., Gaithersburg, MD) with program A-27 according to the manufacturer's protocol as described previously.[20] After transfection, cells were cultured with and without DT3 addition at 37°C with 5% CO_2 until irradiation on the next day (24 h after siRNA transfection). Western blots and colony assays were performed 24 h post-irradiation (48 h after siRNA transfection).

Immunofluorescence staining

Sectioned mouse sternums from DT3- and vehicle control-treated mice 24 h after TBI, and cytospin slides from DT3- and vehicle control-treated human CD34+ cells 4 h post-irradiation were processed for immunostaining. Specimens were incubated in blocking buffer (5% BSA in PBS), followed by primary antibodies (anti-p-Erk1/2 and anti- γ-H2AX), and FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-goat IgG second antibodies. Slides were rinsed in high salt PBS (0.4M NaCl in PBS), counterstained for DNA with 4, 6-diamidino-2-phenylindole (DAPI) mounting medium, and coverslipped. Slide images were examined with a ZEISS fluorescence microscope using the AxioVision MTB2004 configuration.

Statistical analysis.

The difference in mouse 30-day survival was analyzed using Fisher's exact test. Differences between means were compared by ANOVA and Student's *t* tests. $P<0.05$ was considered statistically significant. Results are presented as means ± standard deviations or standard errors of the mean as indicated.

Results

DT3 enhanced mouse survival after γ-irradiation and protected mouse bone marrow hematopoietic progenitors

Figure 1A shows a typical survival study.[19] A single subcutaneous (sc) injection of DT3 (400 mg/kg) 24 h before TBI (8.75 Gy, Cobalt 60 γ-radiation, 0.6 Gy/min) protected CD2F1 mice from radiation-induced mortality with 100% thirty-day survival. In contrast, radiation-induced death in control vehicle-injected mice started 8 days post-irradiation and only 18% survival was

obtained 30 days after TBI (N=16). The time course for vehicle controls shown in Figure 1A is similar to what we have observed in other studies, and is consistent with mortality due largely to hematopoietic injury.[19,21]

We further determined the effects of DT3 on γ-irradiated mouse bone marrow hematopoietic progenitor and stem cell survival (Figure 1B, N = 6). Bone marrow cells were collected from femurs and humeri on days 1, 8 and 13 after irradiation and total live bone marrow myeloid cells for pooled samples from each mouse were measured by Trypan blue staining. _-irradiation caused mouse bone marrow cell death within 24 h, resulting in total live cell numbers decreasing from 7.6 ± 2.5 $x10^6$ cells/mouse before irradiation to 2.3 ± 1.2 $x10^6$ cells/mouse after irradiation ($P<0.01$). DT3 administration at either -24 h or +6 h with respect to irradiation did not significantly change the total live cell numbers.

Eight days after irradiation, one out of 6 mice in the vehicle-treated, TBI group was dead, and the surviving mice exhibited only 10.4±8.5% cell viability (Annexin-V and 7AAD negative, Supplement figure 1A) with 0.8±1.2 x 10^4 total live cells/mouse. In contrast, no animal deaths occurred in DT3-treated mice by day 8. DT3 injection (-24 h) resulted in 33±7.9% total bone marrow cell viability (Supplementary figure 1A) with 19±6 x 10^4 and 11±4x10^4 total live cells/mouse in -24 h and +6 h DT3-treated mice, respectively ($P<0.01$, Figure 1B upper panel).

Thirteen days after irradiation, DT3 administrated at either -24 h or +6 h increased mouse bone marrow myeloid cell numbers to 3.17± 0.7 x 10^6 and 2.06±0.9 $x10^6$, respectively. In the vehicle control group, 40% of mice survived as shown in figure 1A, with a very low proportion of live bone marrow cells (Figure 1B).

The phenotypes of mouse bone marrow cells after DT3 or vehicle treatment, 24 h post-irradiation, were determined by flow cytometric assays. Interestingly, DT3 treatment resulted in significant increases in Lin-/Sca-1+/ckit+ stem and progenitor cells in marrow regardless of irradiation (Supplementary figure 1B). In addition, clonogenicity was compared between samples collected from individual mice treated with DT3 or vehicle at +6 h. Although DT3 did not enhance total bone marrow live cells 24 h after irradiation, DT3 administration resulted in total colony number increases from 27 ± 5 x 10^3/mouse (vehicle-treated mice) to 48±6x10^3/mouse (DT3-treated mice) in non-irradiated samples ($P<0.01$), and from 12±2x10^3/mouse (vehicle-treated) to 32 ± 10 x10^3/mouse (DT3-treated) in samples from mice that had been irradiated (8.75 Gy, $P<0.01$), as shown in Supplementary figure 1C.

Bone marrow pathological changes in sectioned sternum samples from DT3-treated and control animals at 1 and 8 days post-irradiation were evaluated. Marrow cellularity was measured in microscopic fields for each sectioned sternum as described in Design and Methods. Consistent with total bone marrow cell counts, severe bone marrow cellular failure was observed in vehicle-treated mice 24 h after γ-radiation. The effects of DT3 on bone marrow cell survival were observed but were not significant 24 h post-irradiation (data not shown). We further evaluated the bone marrow 8 days after irradiation (Figure 1C). Cellularity in vehicle control mice decreased from 29% at 24 h to 4% on day 8. Cellularity in DT3-treated mice decreased from 42% at 24 h to 14% on day 8. Compared to the non-irradiated mouse bone marrow (Figure 1C: panel 1, 20x, and panel 4, 200x), lethal irradiation reduced marrow cellularity, resulting in no erythroid, rare myeloid, and zero megakaryocytic cells (Figure 1C: panel 2, 20x and panel 5, 200x). However, in DT3-treated mice (-24 h), regenerative microfoci were present (Figure 1C: panel 3, 20x and panel 6, 200x) in the ends of the sternebrae. In DT3-treated mice, regenerative

microfoci accounted for up to 50% cellularity per low-power (200x) field in ends of sternebrae. Significant megakaryocyte restoration occurred in DT3-treated mice 8 days post-irradiation (Figure 1C: panel 6).

DT3 protected human CD34+ cells from radiation damage

Consistent with the *in vivo* mouse study, DT3 (2 μM) addition 24 h prior to _-irradiation in human hematopoietic CD34+ cells (*in vitro*) increased cell survival 1.6 and 2.4 fold 3 days after 2 or 4 Gy irradiation ($P<0.05$, Figure 2A). Hematopoietic progenitor granulocyte-macrophage colony forming units (CFU-GM, Figure 2B) in irradiated human CD34+ cells were markedly enhanced by DT3, but the erythroid lineage (BFU-E and CFU-E) colony numbers were not changed by DT3. Unlike the *in vivo* study, survival effects of DT3 added 6 h after irradiation were not observed in CD34+ cells (data not shown).

DT3 induced Erk phosphorylation and reversed the inhibition of mTOR and downstream effector activation by irradiation in human CD34+ cells

We next evaluated DT3-induced stress-response signal regulation in human CD34+ cells. Immunoblotting data showed that addition of DT3 dramatically induced Erk phosphorylation in CD34+ cells regardless of irradiation (Figure 3A). This result was confirmed by immunofluorescence staining using anti-human phospho-Erk1/2 (p-Erk)-FITC antibody as shown in Figure 3B. DT3 treatment resulted in Erk phosphorylation in 90% of unirradiated CD34+ cell nuclei. In contrast, untreated human CD34+ cells possessed little or no Erk phosphorylation, and γ-radiation did not induce Erk phosphorylation in these cells (Figure 3A).

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Results from our immunoblotting data showed mTOR was highly expressed in cultured CD34+ cells. However, levels of mTOR, as well as downstream effector ribosomal protein S6 (S6) expression and phosphorylation, decreased after γ-irradiation (figure 3C). mTOR plays a central role in regulating protein synthesis, ribosomal protein translation, and cap-dependent translation.[12] Major upstream signaling components that control mTOR activity are the PI3K/PTEN/Akt and Ras/Raf/Mek/Erk pathways.[14,15] Interestingly, addition of DT3 dramatically induced Erk phosphorylation and reversed the radiation-induced inhibition of mTOR and S6 protein activation in CD34+ cells 4 h after 2 Gy irradiation. In contrast, Akt protein expression and phosphorylation were undetectable (Figure 3C), suggesting DT3 specifically influences Erk signaling. Another direct target of mTOR is 4EBP1, which inhibits the ability of eIF4E to form complexes with eIF4G and mediate cap-initiated mRNA translation in mammalian cells.[22, 23] mTOR inactivates 4EBP1 through the induction of 4EBP1 phosphorylation, which results in eIF4E release from 4EBP1 binding, and consequently stimulates cap-dependent mRNA translation. In addition, the activation of 4EBP1 can be controlled by the MAP kinase-Erk pathways.[16, 24] Both mTOR and Erk inhibit 4EBP1 through phosphorylation of its regulatory sites. To evaluate the consequences of DT3-induced mTOR and Erk activation, we examined 4EBP1 phosphorylation in DT3-treated human CD34+ cells. The Western blots in Figure 3D show addition of DT3 dramatically induced 4EBP1 protein phosphorylation and expression regardless of irradiation. In vehicle control-treated cells, 2 Gy γ-irradiation did not significantly upregulate 4EBP1 phosphorylation although the total 4EBP1 protein level was increased.

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DT3 upregulated Erk and mTOR signal pathway activation in mouse marrow cells 4 or 24 h after γ-irradiation

To investigate whether DT3 also induces Erk, mTOR, S6 activation and 4EBP1 phosphorylation in the *in vivo* mouse model, we evaluated individual bone marrow samples. Since 8.75 Gy irradiation caused massive bone marrow cell death, resulting in insufficient material for immunoblotting, we collected samples from mice irradiated at 5 Gy. Western blots show phosphorylated Erk, mTOR, and S6 expression in marrow from DT3-treated mice 4 and/or 24 h after 5 Gy irradiation (Figure 4A). Phospho-Erk was expressed in one out of three DT3-treated marrow samples 4 h after irradiation and in all three DT3-treated samples 24 h after irradiation. Comparative immunofluorescence staining showed similar results in mouse sternum marrow slides. In these assays, anti-mouse p-Erk1/2 was labeled with Rhodamine (red). Figure 4B shows Erk was very weakly phosphorylated in vehicle-treated marrow cells 24 h after 5 Gy γ-irradiation. However, upregulation of p-Erk was observed in 90% of marrow cells from DT3-treated, 5 or 8.75 Gy-irradiated mice. The data shown in Figure 4C indicate a significant upregulation of Erk phosphorylation in DT3-treated, irradiated mice (N=6/group). Unlike in human CD34+ cells, mTOR expression and phosphorylation were observed only in DT3-treated and irradiated mouse bone marrow cells. Marrow contains a mixture of cell types, and levels of mTOR expression may vary in these cells. Akt protein expression was observed in mouse bone marrow cells, but Akt phosphorylation was not detectable. Consistent with human CD34+ cells, DT3 induced S6 phosphorylation in marrow samples with and without irradiation. Furthermore, 4EBP1 protein phosphorylation was observed in all DT3-treated marrow samples 24 h post-irradiation, correlated with eIF4E activation (Figure 4D). Interestingly, one bone marrow sample

from the vehicle control-treated, irradiated group displayed 4EBP-1 phosphorylation and eIF4E activation.

Erk gene knockdown suppressed m-TOR phosphorylation and clonogenicity and increased γ-H2AX foci formation in DT3-treated CD34+ cells after IR

Erk is one of the major upstream signaling components controlling mTOR activity.[14, 15] To verify that DT3 stimulation of the mTOR pathway results from Erk activation, *Erk1/2* siRNA was transfected into CD34+ cells before DT3 addition and γ-irradiation using nucleofector technology.[20] Western blots and colony assays were performed 24 h after irradiation, 48 h after *Erk* siRNA or control siRNA (maxGFP siRNA) transfection or non-gene transfection with DT3 supplementation. Results from Western blots showed Erk1/2 protein levels markedly decreased after *Erk* siRNA transfection (Figure 5A). In contrast, control siRNA-transfected cells expressed Erk1/2 at the same level as nontransfected samples. As expected, Erk1/2 knockdown blocked mTOR phosphorylation in DT3-treated CD34+ cells after irradiation (Figure 5B). Furthermore, clonogenicity in DT3-treated CD34+ cells was significantly inhibited by *Erk* gene knockdown compared with control siRNA-transfected and non-gene-transfected samples after 2 and 4 Gy irradiation (Figure 5C).

The primary target of radiation-induced cell death in eukaryotes is generally considered to be DNA, although protein oxidation may also trigger signals leading to cell death.[25] Double strand breaks (DSBs) are the major lethal lesions.[26, 27] To verify that DT3-stimulated Erk and mTOR activation is associated with its radiation countermeasure function, we further examined the interaction between phosphorylation of Erk expression and DNA-damage signal γ-H2AX focus

formation[28] in human CD34+ cells using immunofluorescence staining with p-Erk1/2 and γ-H2AX antibodies. In figure 6, anti-human γ-H2AX-Rhodamine (red) is shown with anti-human p-Erk1/2- FITC (green). _-H2AX foci were formed in vehicle control nuclei: 7.65 ± 3.05 foci per cell, 4 h after 2 Gy irradiation (Figure 6A). Phospho-Erk expression in these nuclei was undetectable. In contrast, DT3 addition (Figure 6B) upregulated p-Erk expression in 90% of irradiated cells and decreased γ-H2AX focus formation to 0.82±1.03 per-cell 4 h after 2 Gy irradiation ($P<0.001$, DT3-treated vs. vehicle-treated cells). Finally, γ-H2AX foci were measured in Erk siRNA-transfected and DT3-treated cells to test whether this would reverse protection by DT3 (Figure 6C). Consistent with clonogenicity results (Figure 5C), Erk knockdown abrogated the effects of DT3 on protection of DNA-DSBs in CD34+ cells after γ-irradiation, as reflected in the number of γ-H2AX foci.

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Discussion

We demonstrated that natural DT3 from palm oil or rice bran oil protected mice from a lethal dose of total body γ-irradiation and resulted in 100% 30-day survival compared with 18% survival in vehicle-treated control mice. Exposure to a lethal dose of IR decreased viable cells in bone marrow. DT3 administration significantly increased cell viability on day 8 and further improvements were obtained on day 13. Attempts were made to replicate the *in vivo* studies in human CD34+ cells so that molecular mechanisms could be studied in greater detail. Results (Figure 2) revealed that DT3 added to cell culture 24 h prior to radiation increased cell viability as well as specifically stimulating CFU-GM colonies. The doses of radiation chosen for CD34+ were similar to those used in our previous publications.[20]

The basis of the present study was to understand the mechanism behind DT3 radioprotection in mice and human CD34+ cells. We found DT3 administration induced Erk1/2 phosphorylation in mouse bone marrow and human CD34+ cells, and resulted in significant inhibition of the DNA-DSB marker γ-H2AX focus formation in CD34+ cells after γ-irradiation. Erk activity is a component of the pro-survival MAPK pathway, which induces expression of DNA repair and cell growth factors.[9, 10] However, normal human CD34+ cells have been reported to possess little or no Erk phosphorylation,[29, 30] and our data (Figure 3A and B) confirm the lack of Erk phosphorylation. Therefore, we asked whether DT3-induced Erk phosphorylation is associated with DT3's radioprotective function in mice and in human hematopoietic cells. The reason for the preferential stimulus of the granulocyte-macrophage (GM) lineage by DT3, as shown in clonogenic assays (Figure 2B), is not known. However, a recent report [30] suggests that the MEK-ERK signaling plays a critical role in regulation of expansion of myeloid precursor cells through

up-regulation of the cyclin D1 gene. Since DT3 induces ERK phosphorylation, this might result in generation of GM progenitor cells.

We also found mTOR was highly expressed and activated in cultured CD34+ cells (Figure 3C), and that irradiation led to the inhibition of mTOR in these cells. Consistent with our results, Braunstein et al.[24] recently reported that in ionizing radiation-sensitive (non-transformed) cells, radiation inhibits cap-dependent protein synthesis through a mechanism that involves inhibition of mTOR and maintained hypophosphorylation of 4EBP1, which binds avidly to eIF4E and inhibits cap-dependent mRNA translation initiation. We found DT3 reversed IR-induced suppression of mTOR activation, resulting in 4EBP1 phosphorylation and S6 and eIF4E activation (Figure 5 D). Both 4EBP1 and S6 kinase are mTOR targets, which initiate mRNA translation in mammalian cells.[31] The phosphorylation of 4EBP1 can also be directly controlled by the MAPK/Erk pathway.[16,24] Translational modulation is more rapid than transcriptional modulation in response to positive and negative stimuli including radiation stress.[22] Regulation of protein synthesis in eukaryotes through mRNA translation plays a critical role in cell cycle, proliferation, differentiation, and apoptosis. Evidence from our *in vivo* studies presented in Figure 4D indicated one bone marrow sample from vehicle control-treated and irradiated mice displayed 4EBP-1 phosphorylation and eIF4E activation. DT3 up-modulated 4EBP-1 phosphorylation and enhanced mouse bone marrow survival. The data suggest the possibility that inter-individual variability in baseline activation of mTOR pathways may be related to variations in survival of irradiated, untreated mice.

There are two major upstream regulators of mTOR: Akt and Erk1/2.[14,15] In the present study, DT3 upregulated Erk1/2 and mTOR, but not Akt activation, in mouse bone marrow and human hematopoietic progenitor cells. To determine whether DT3-induced mTOR activation is through

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Erk regulation, we inhibited Erk1/2 gene expression in CD34+ cells using siRNA and found Erk1/2 knockdown significantly blocked the effects of DT3 on mTOR activation and survival of progenitors. Redon et al[28] recently reported a linear response proportion between the initial γ-radiation dose and γ-H2AX foci formation in blood cells and suggested using γ-H2AX foci formation to monitor DNA damage in blood and skin cells after γ-irradiation. The correlation between Erk phosphorylation and radiation-induced DNA damage was assessed in CD34+ cells using γ-H2AX expression (Figure 6). It is clearly evident from the irradiated, Erk gene knockdown sample (Figure 6 C) that Erk phosphorylation and γ-H2AX foci formation are inversely correlated.

Previous studies suggest the tocotrienols' anticancer properties are through apoptotic mechanisms and regulation of PI3K/Akt, NFkB, and MAPK signaling.[32,33] Further, many research reports revealed that tocotrienols induced apoptosis preferentially in cancer cells but not in normal cells,[34] although the mechanisms are not understood. Erk, an anti-apoptotic factor, may participate in the transmission of many mitogenic and oncogenic signals that lead to the accelerated proliferation observed with malignant transformation.[35] Although DT3 induces Erk phosphorylation, the induction is transient since Erk and mTOR phosphorylation disappeared in day 13 samples (data not shown). Hence this effect may not be equivalent to the DT3 effect on cancer cells.

Radioresistant mechanisms are frequently acquired by tumor cells with constitutive activation of NFkB,[36] Erk1/2,[37] and Akt. [38] However, in healthy hematopoietic cells, especially in immature progenitor and stem cells, expression and activation of these survival proteins are relatively low. We reported that IR induced degradation of pro-survival factor NFkB subunit p50 in CD34+ cells, which was associated with a lack of NFkB activation, and may be partly responsible for the

radiation sensitivity of CD34+ cells.[20] Results from the present study and others[29] demonstrated that normal human hematopoietic CD34+ cells have little or no Erk phosphorylation, and our data further show that phosphorylated Erk expression was very low or undetectable after γ-irradiation in mouse bone marrow and human CD34+ cells. Here, studies in mouse bone marrow and human CD34+ cells indicate Erk/mTOR signaling was necessary for DT3's radioprotective efficacy, hence this molecular pathway should be explored as a possible target of radiation countermeasure candidates.

In conclusion, our data indicate that DT3 as a pro-proliferative and anti-apoptotic agent inhibits radiation-induced DNA-DSBs in hematopoietic stem and progenitor cells and protects mice and human CD34+ cells from radiation-induced mortality through increase of bone marrow regeneration capacity and hematopoietic stem and progenitor cell proliferation. The mechanism of DT3-mediated radioprotection may be attributed to its stimulation of Erk activation-associated mTOR survival pathways.

Authorship and Disclosures

XHL, DF, and NHL performed the *in vivo* and *in vitro* research work and analyzed the data. CPM and PHN prepared dugs and injected the mice. SRM performed the mouse bone marrow pathology study, and collected and analyzed data. VS designed the mouse survival study, analyzed the data and wrote the paper. MHW analyzed the data and wrote the paper. MX was the principal investigator, designed the research, analyzed the data and wrote the paper, and takes primary responsibility for the paper.

The authors reported no potential conflicts of interest.

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Legends for figures

Figure 1. DT3 protects mouse bone marrow hematopoietic cells after gamma-irradiation. (A) Protective effect of DT3 on survival of irradiated CD2F1 mice (*n* =16). DT3 or vehicle sc 24 h before TBI (8.75 Gy at dose rate 0.6 Gy/min). The difference in 30-day survival between vehicle-injected and DT3-injected groups was statistically significant (P<0.01). (B) Total live cell counts of mouse bone marrow myeloid cells from pooled femur and humerus samples from vehicle control (N=6) and DT3-treated (-24 h or +6 h irradiation) mice (N=6) 1, 8, and 13 days post-irradiation. Total live cell counts of mouse bone marrow myeloid cells 8 days are shown in upper panel (y axis: 10^4/mouse). Means±SD. **: P<0.01, DT3-treated vs. vehicle-treated mice. (C) HE staining of bone marrow from DT3-treated and control mice 8 days post-irradiation (8.75 Gy): longitudinal sections of entire sternum from representative mice in different groups at 20X magnifications are shown in upper panels (panels 1, 2, and 3). Sections in bottom panels (4, 5, and 6) present higher magnifications of the selected areas of each corresponding upper panel indicated by rectangles (200X). Megakaryocyte foci are identified with arrows.

Figure 2. DT3 protected human CD34+ cells (*In vitro*) from radiation damage. DT3 (2 μM) was added 24 h before irradiation. Following irradiation (0, 2, or 4 Gy), cell expansion (A) and colony formation (B) assays were performed. Total cell numbers from each group (N=3) were counted 3 days after irradiation, and colonies were counted 14 days later. Results are from a total of three experiments and each experiment was performed in triplicate. Means±SD. *:*P*<0.05, **:*P*<0.01, DT3 vs. control (CT) culture.

Figure 3. Effects of DT3 on Erk, activation of mTOR and S6, and 4EBP-1 phosphorylation in CD34+ cells. (A) Western blot shows total Erk1/2 protein and p-Erk1/2 expression in DT3- or vehicle-treated CD34+ cells 4 h after 2 and 4 Gy-irradiation. β-actin served as internal loading control. (B) Immunofluorescence staining shows p-Erk1/2 (green) expression in unirradiated CD34+ cells 24 h after DT3 addition. DAPI (blue) defines cell nuclei. (C) m-TOR, S6, and AKT expression and phosphorylation in vehicle control- or DT3-treated CD34+ cells were determined by Western blot 4 h after 2 Gy irradiation. (D) Western blot: 4EBP-1 was detectable in all samples, and expression and phosphorylation were significantly increased in DT3-treated samples. Shown are representative immunoblots and statistical data from three experiments. Means ± SD. *:$P<0.05$, **:$P<0.01$. 0 Gy *vs.* 2Gy or DT3-treated vs. vehicle-treated.

Figure 4. DT3 stimulated Erk, mTOR, S6, and eIF4E activation, and 4EBP-1 phosphorylation in mouse bone marrow**.** Mice injected with vehicle or DT3 24 h prior to irradiation (N=6/group). Total bone marrow cells from each femur and humerus were collected 4 or 24 h post-irradiation. (A) Samples were analyzed by immunoblotting. Erk, mTOR, and S6 phosphorylation were upregulated by DT3 4 and/or 24 h after 5 Gy irradiation. Akt expression but not phosphorylation was observed in all samples. (B) Mouse sternums were fixed with Z-Fix and stained using immunofluorescent anti-mouse p-Erk-Rhodamine (red) 24 h after 5 or 8 Gy irradiation. DAPI (blue) defines cell nuclei. Slides were examined with a ZEISS fluorescence microscope using AxioVision MTB2004 configuration. Original magnification is X630. (C) p-Erk levels were quantified against total Erk protein amounts. The results represent means±SD **: P<0.01. (N=6 mice). (D) 4EBP-1 and eIF4E were measured by immunoblotting. Radiation upregulated 4EBP-1 in marrow cells from both vehicle control- and DT3-injected mice. In 5 Gy irradiated mice,

phosphorylation of 4EBP-1 and eIF4E were observed in one vehicle-treated sample and all samples from DT3-injected mice. β-actin served as internal loading control.

Figure 5. *Erk* gene knockdown in CD34+ cells after IR. (A) SignalSilence Erk1 and Erk2 siRNA from siGENOME SMARTpool (1.5 μg each) or 1.5 μg of maxGFP siRNA (positive control) were transfected into 3 x 10^6 human CD34+ cells. Western blots were performed 48 h post-siRNA transfer. (B) *Erk1* gene knockdown suppressed m-TOR phosphorylation in DT3-treated CD34+ cells 24 h after 2 or 4 Gy _-irradiation. Shown are representative immunoblots and statistical data from three experiments. Means±SD. (C) DT3-induced clonogenicity was significantly reduced by *Erk* gene knockdown in CD34+ cells after 2 Gy irradiation. Results are from a total of two experiments and each experiment was performed in triplicate. Means ± SD. *: P<0.05, **:P<0.01. si-CT *vs*. si-Erk. (D) Schematic diagram of the role of DT3 in radioprotection. DT3 reversed IR-induced suppression of mTOR activation, resulting in 4EBP1 phosphorylation and S6 and eIF4E activation, which initiates mRNA translation and results in hematopoietic cell survival and growth. The phosphorylation of 4EBP1 can also be directly controlled by the MAPK/Erk pathway.

Figure 6. Effects of DT3 on human CD34+ cell DNA-DSBs after IR are Erk-dependent. Immunofluorescence staining using anti-human- γH2AX-Rhodamine (red) and anti-human-phospho-Erk-FITC (green) antibodies in DT3- and control-treated CD34+ cells 4 h after 2 Gy γ-irradiation. Radiation induced γ-H2AX expression (A) in control-treated cells, which expressed p-Erk at undetectable levels. In contrast, DT3 induced p-Erk expression in 90% of cells (B) and decreased γ -H2AX focus formation. Knockdown of *Erk1/2* gene (C) resulted in increased γ-

H2AX foci in DT3-treated CD34+ cells. Average numbers of γ-H2AX foci per cell were counted in 50-100 cells per sample as shown at the bottom left corners of the γ-H2AX-Rhodamine images (Means±SD. $P<0.001$, DT3-treated *vs.* vehicle-treated and DT3-treated vs. Erk1/2 knockdown/DT3-treated). DAPI (blue) defines cell nuclei. Slides were examined with a ZEISS fluorescence microscope using AxioVision MTB2004 configuration. Original magnification is X630.

Supplementary Figure 1. DT3 protects mouse bone marrow hematopoietic cells after γ-irradiation. Mice were exposed to 8.75 Gy γ-irradiation, 0.6 Gy/min. Total live cell counts of mouse bone marrow myeloid cells from pooled femur and humerus samples are from vehicle control (N=6) and DT3-treated (-24 h or +6 h irradiation) mice (N=6). (A) Viability measured as percentage of Annexin-V- and 7AAD-negative cells in individual mouse 8 days post-irradiation and (B) Phenotypes of bone marrow cells analyzed by FACSCalibur. Lin-/c-kit+/Sca-1+ cell populations were identified. (C) Clonogenicity of mouse bone marrow cells that received DT3 or vehicle treatment 6 h post-irradiation (N=6/group) was quantitated. Total colonies were counted 10 days after plating and are expressed as colonies per mouse. Means ± SD. **: P<0.01, DT3-treated *vs.* vehicle-treated.

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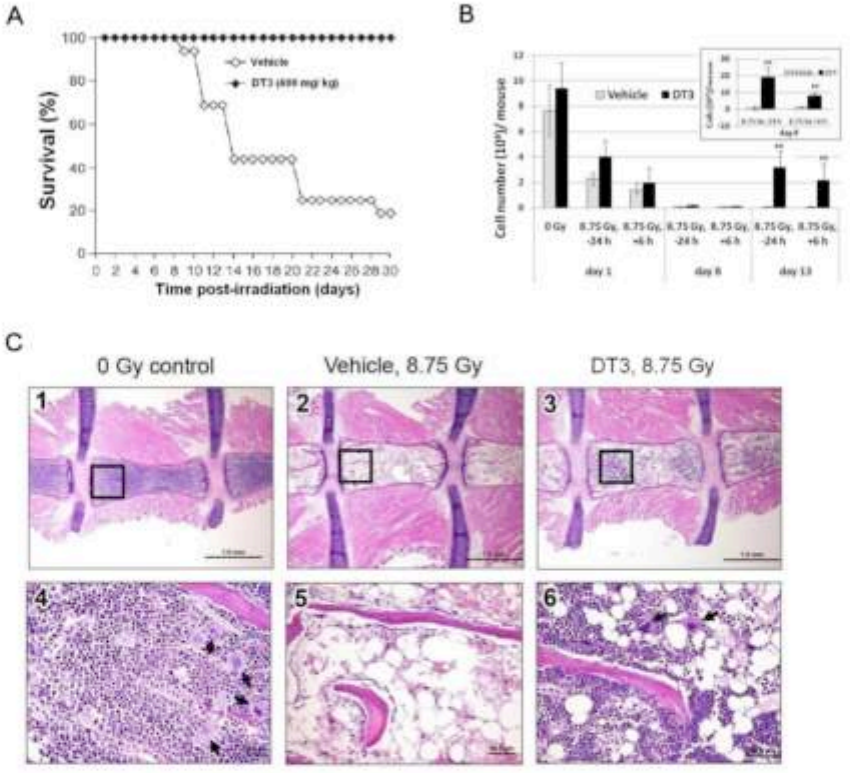

Figure 1A-C, Xiao

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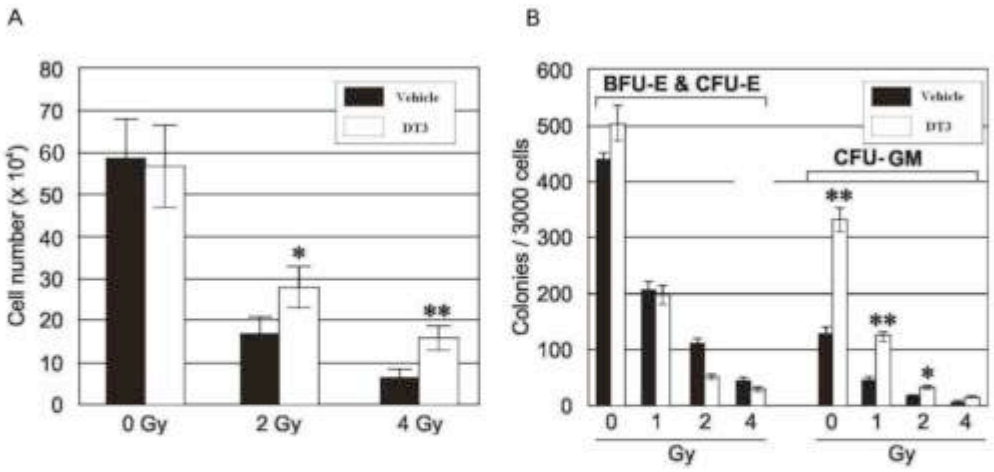

Figure 2 A& B, Xiao

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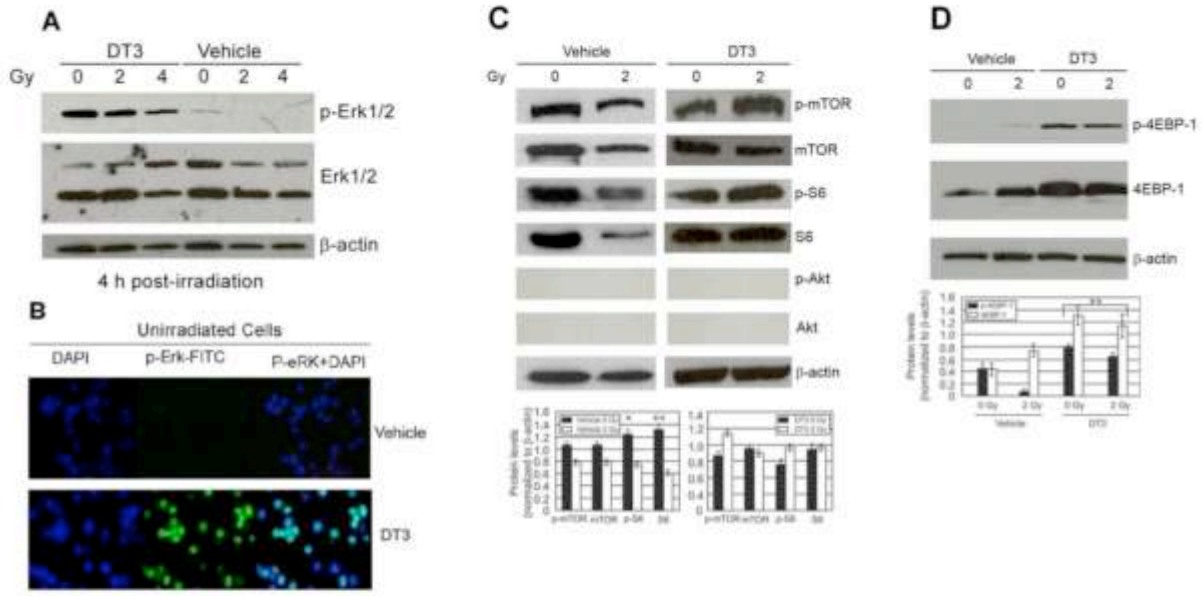

Figure 3 A-D-Xiao

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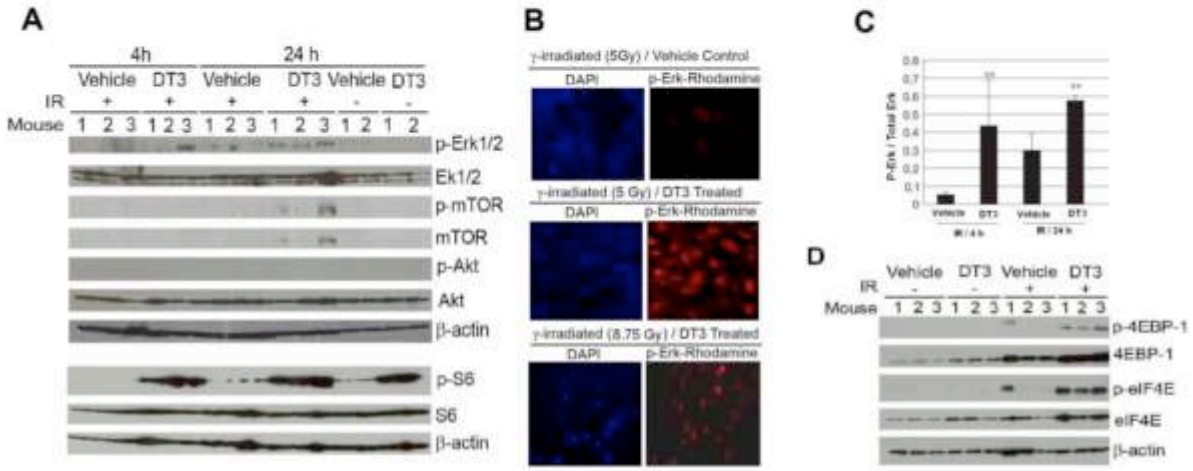

Figure 4 A-D, Xiao

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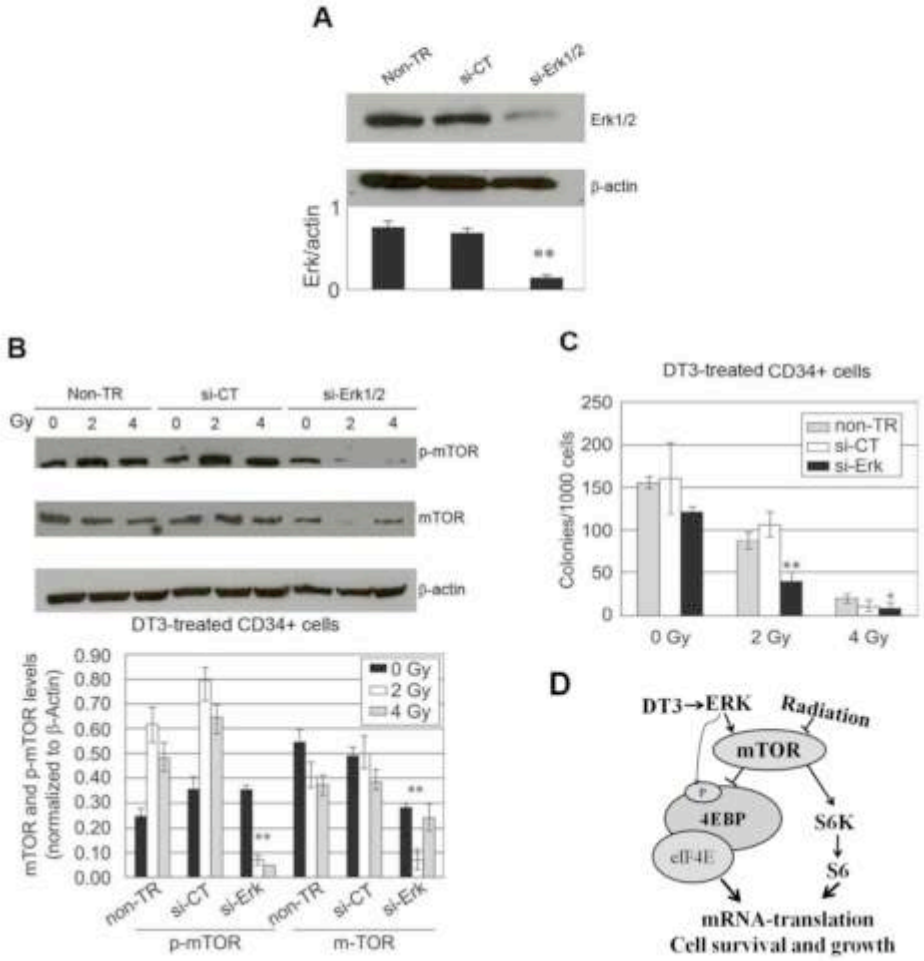

Figure 5 A-D, Xiao

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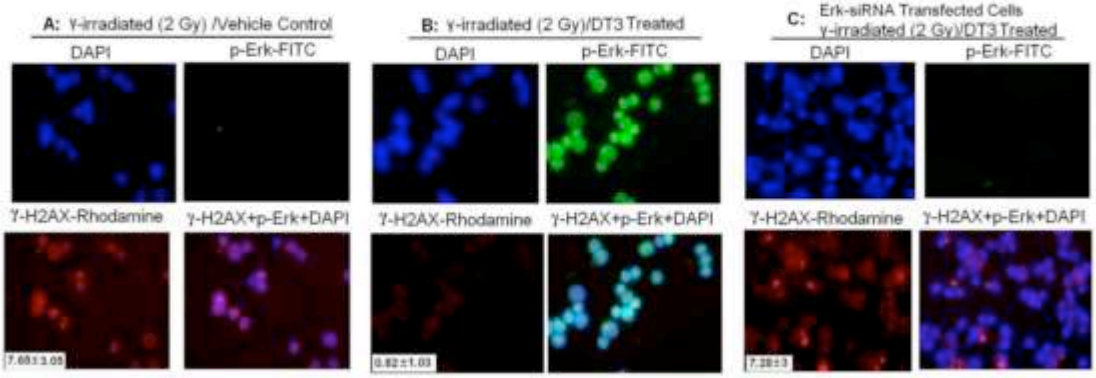

Figure 6 A-C, Xiao

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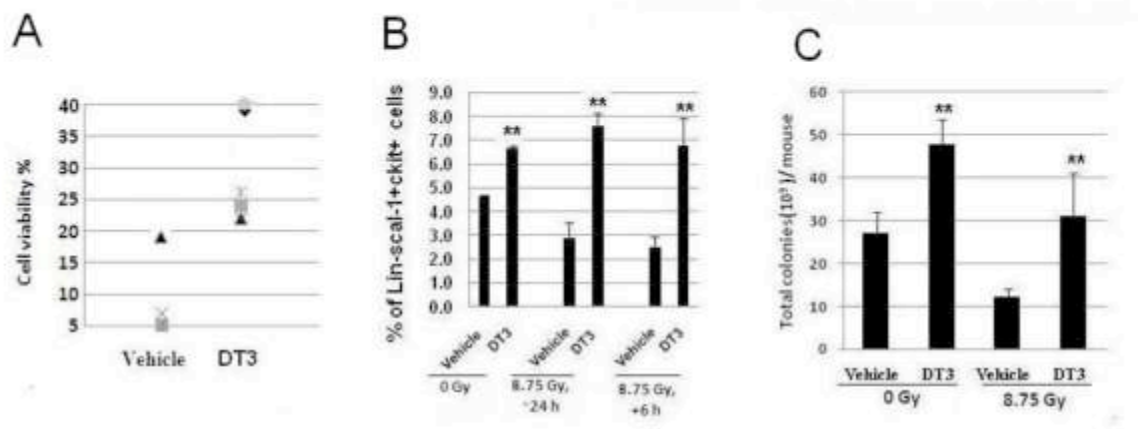

Supplementary Figure 1A-C, Xiao